Toll-Like Receptor Adaptor Molecules Enhance DNA-Raised Adaptive Immune Responses against Influenza and Tumors through Activation of Innate Immunity

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Toll-like receptors (TLRs) recognize microbial components and trigger the signaling cascade that activates the innate and adaptive immunity. TLR adaptor molecules play a central role in this cascade; thus, we hypothesized that overexpression of TLR adaptor molecules could mimic infection without any microbial components. Dual-promoter plasmids that carry an antigen and a TLR adaptor molecule such as the Toll–interleukin-1 receptor domain-containing adaptor-inducing beta interferon (TRIF) or myeloid differentiation factor 88 (MyD88) were constructed and administered to mice to determine if these molecules can act as an adjuvant. A DNA vaccine incorporated with the MyD88 genetic adjuvant enhanced antigen-specific humoral immune responses, whereas that with the TRIF genetic adjuvant enhanced cellular immune responses. Incorporating the TRIF genetic adjuvant in a DNA vaccine targeting the influenza HA antigen or the tumor-associated antigen E7 conferred superior protection. These results indicate that TLR adaptor molecules can bridge innate and adaptive immunity and potentiate the effects of DNA vaccines against virus infection and tumors.

Vaccines have played a substantial role in controlling epidemic diseases since Jenner's innovation. However, despite the competency of current biomedical sciences, some pathogens remain in place and pose a serious threat. Although DNA vaccines have been highlighted for their potential to conquer such uncontrolled pathogens, the magnitude of the immune response elicited by DNA vaccines in larger animals, including humans, has been disappointing (3). Numerous strategies have been explored to improve their immunogenicity, ranging from the use of gene guns and electroporation to improve vaccine uptake, to the coadministration of cytokines and chemokines to boost local immunity, to the use of "combination" vaccines that boost immunity with protein- or vector-based components (13). One promising strategy is the use of adjuvants that enhance DNA-raised immune responses (17).

One important class of immune adjuvants triggers the innate immune system via Toll-like receptors (TLRs) (10, 19). These agents mimic infection by microbes, thereby eliciting the innate immune responses required for subsequent acquired immune activation against immunogens. However, the strong immunostimulatory properties of TLR ligands may induce unwanted side effects, ranging from local irritation to systemic shock (2). TLR ligands may also exacerbate preexisting neuronal or autoimmune diseases (7, 14, 16). To circumvent these complica-

tions, we examined whether TLR adaptor molecules in cells transfected together with DNA vaccines augment adaptive immune responses while avoiding adverse reactions.

TLR-mediated cell activation proceeds through two distinct pathways. The first pathway depends on an adaptor molecule, myeloid differentiation factor 88 (MyD88), that transduces downstream events leading to nuclear factor κB (NF-κB) activation (27). MyD88 associates with the proximal TIR domains of TLRs, initiating a signaling cascade that involves activation of interleukin-1 (IL-1) receptor-associated kinase (IRAK) family members and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (28). This pathway is triggered by TLR2, TLR4, TLR5, TLR7, TLR8, and TLR9 (26). In the case of TLR7 and TLR9, the MyD88-dependent pathway also triggers phosphorylation of interferon regulatory factor 7 (IRF-7) by IRAK1, which leads to alpha interferon (IFN-α) production (35). Toll–IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) and the TRIF-related adaptor molecule (TRAM) mediate a MyD88-independent alternative pathway. Once TRIF binds to activated TLR3 or TLR4, it interacts with TRAF6, inducible IKK, and TANK-binding kinase 1, mediating the induction of IFN-β through the activation of NF-κB and IRF-3 (8). Overexpression of these adaptor molecules is proven to turn on downstream cellular signaling in the absence of TLRs or TLR ligands (11, 29); thus, we intended to use TLR-adaptor molecules as genetic adjuvants.

The present study shows that DNA vaccine immunogenicity is enhanced by cotransfection of MyD88 and TRIF. The TRIF genetic adjuvant improved the protective effect of DNA vaccines against lethal influenza virus challenge and tumor out-

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growth, a result that may have an impact on current vaccine development targeting emerging viral infections and tumors.

MATERIALS AND METHODS

Expression plasmids. cDNA fragments encoding immunogens and TLR adaptor molecules were amplified from parental plasmids by PCR and subcloned into the pGA vector. The FLAG-tagged expression plasmids CMV4-MyD88, CMV4-TRIF, CMV4-TIRAP, CMV4-TOLLIP, CMV4-IRAK1, and CMV4-TRAF6 have been described previously (29). LacZ or EGFP cDNA was amplified from pSV-β-Galactosidase (Promega, Madison, WI) or pEGFP-N1 (BD Biosciences, San Jose, CA), respectively. Influenza virus A/PR/8/34 (H1N1) HA (amino acids 18 to 566) was amplified from pJW4303/H1 and introduced into the pFLAG-CMV9 vector (Sigma, St Louis, MO). cDNA encoding $E7_{49}$ attached to the carboxy terminus of EGFP was cloned into pGA vector (4). Sequences of cloned PCR products were confirmed with an ABI PRISM Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

Cell transfection and reporter gene assay. Transient transfections were conducted with FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. HEK293 cells (3×10^4) were cotransfected with each expression plasmid (CMV4, CMV4-MyD88, CMV4-TRIF, CMV4-TIRAP, CMV4-TOLLIP, CMV4-IRAK1, CMV4-TRAF6, pGA-LacZ, pGA-LacZ-MyD88, or pGA-LacZ-TRIF [200 ng]), the reporter plasmid (p5xNF-κB-luc or pGL3 hIFNβ [25 ng]), and the control *Renilla* luciferase (LUC) plasmid (pTK-RL [25 ng]; Promega, Madison, WI). Cells were incubated for 48 h after transfection, and LUC activity was measured with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Alternatively, LacZ activity was measured with a β-Gal Reporter Gene Assay kit (Roche Diagnostics). Finally, firefly LUC activity or LacZ activity of individual cell lysates was normalized to *Renilla* LUC activity.

Immunization schedule. Eight-week-old female BALB/c and C57BL/6 mice were purchased from SLC (Shizuoka, Japan) and housed in an animal facility under specific-pathogen-free conditions. After anesthetizing with a ketamine/xylazine mixture, the mice (eight mice/group) were injected in the quadriceps muscles with a plasmid solution (1 μg/μl saline, 50 μl/muscle) containing either pGA-LacZ, pGA-LacZ-MyD88, pGA-LacZ-TRIF, pGA-GFP, pGA-GFP-MyD88, pGA-GFP-TRIF, CMV9-HA, CMV9-HA-TRIF, pGA-GFP-E7, or pGA-GFP-E7-TRIF. The injection site was electroporated with a field strength of 30 V/cm (constant) and three pulses of 50 ms each, by using CUY21EDIT (NepaGene, Tokyo, Japan). The booster immunization was given 2 or 4 weeks after the primary immunization. The institutional animal care and welfare committee approved all of the animal experiments, and the mice were treated in accordance with NIH animal care guidelines.

ELISA. Serum antibody (Ab) titers were measured by enzyme-linked immunosorbent assay (ELISA), as described previously (30).

RT-PCR. Splenocytes were harvested 2 weeks after the final immunization. The cells were incubated with 1 μg/ml of H-2^d-restricted LacZ class I peptide (TPHPARIGL), E7 peptide (RAHYNIVTF), or NP peptide (ASNENMDAM) for 24 h at 37°C. Alternatively, inguinal lymph node (LN) cells were harvested 24 or 72 h after the final immunization. Total RNA was isolated as described previously (31). Real-time PCR (RT-PCR) was performed with TaqMan probes (Applied Biosystems, Foster City, CA) specific for IFN-γ, IL-12 p40, IL-18, IL-4, IL-6, IFN-β, TNF-α, IP-10, JE/MCP-1, major histocompatibility complex class I (MHC-I) (D1), MHC-II (Ea), CD40, CD80, CD86, or 18S rRNA and an ABI PRISM 7700 sequence detection system (Applied Biosystems). The relative mRNA expression levels of IFN-γ, IL-12 p40, IL-18, IL-4, IL-6, IFN-β, TNF-α, IP-10, JE/MCP-1, MHC-I (D1), MHC-II (Ea), CD40, CD80, and CD86 in the individual samples were normalized to 18S rRNA levels.

CTL assay. Splenocytes were harvested 2 weeks after the final immunization. The cytotoxic T-lymphocyte (CTL) isolates (effector cells) were prepared after incubation with 1 μg/ml of H-2^d-restricted LacZ class I peptide and 20 U/ml of IL-2 (Sigma) for 4 days at 37°C. P815 cells pulsed with 1 μg/ml of H-2^b-restricted LacZ class I peptide (DAPIYTNV [control peptide]) or H-2^d-restricted LacZ class I peptide were used as the target cells. The target cells (1×10^4) were cultured with increasing numbers of the effector cells for 4 h. The amount of lactate dehydrogenase released from target cells was measured by the Cytotox 96 (Promega) system, and the percentage of specific lysis was calculated according to the manufacturer's protocol.

Influenza challenge. Two weeks after the booster immunization with pGA-GFP, CMV9-HA, or CMV9-HA-TRIF, the mice were challenged intranasally with 1×10^4 PFU (4 50% lethal doses [LD_{50}]) or 5×10^4 PFU (20 LD_{50}) of influenza virus A/PR/8/34 (21). The body weight and the mortality of the challenged mice were monitored for the next 10 days.
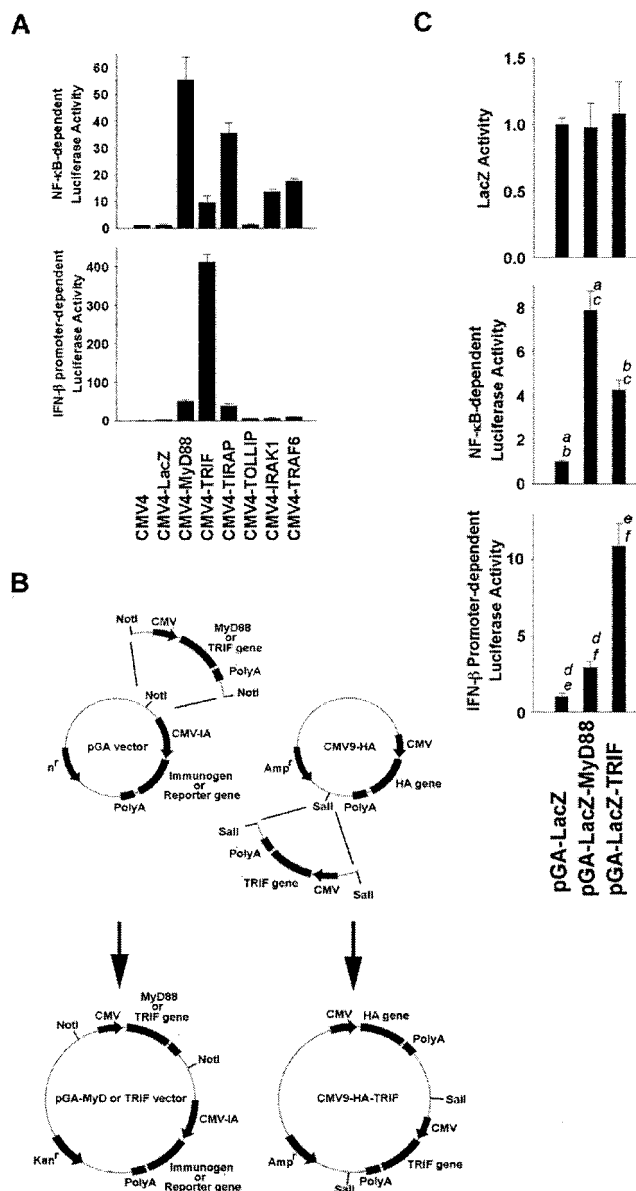

FIG. 1. Characterization of TLR adaptor molecules as activators of the NF-κB and IFN-β promoters. (A) HEK293 cells (4×10^5) were cotransfected with 25 ng of pTK-RL and 25 ng of 5× NF-κB-luc or pGLhIFNβ-luc, plus 250 ng of each indicated expression plasmid for TLR adaptor molecules. Firefly LUC activity was assayed 48 h after transfection and normalized to *Renilla* LUC activity. Values are means ± standard deviations for three independent experiments. The relative LUC activity of cells transfected with 5× NF-κB-luc plus CMV4-MyD88 was significantly higher than that with 5× NF-κB-luc plus any other plasmid ($P < 0.0001$). The relative LUC activity of cells transfected with pGLhIFNβ-luc plus CMV4-TRIF was significantly higher than that with pGLhIFNβ-luc plus any other plasmid ($P < 0.0001$). (B) Schematic diagram of a DNA vaccine expressing a single antigen alone (pGA-LacZ, pGA-GFP, CMV9-HA, and pGA-GFP-E7) or both antigen and adjuvant molecules (pGA-LacZ-MyD88, pGA-GFP-MyD88, pGA-LacZ-TRIF, pGA-GFP-TRIF, CMV9-HA-TRIF, and pGA-GFP-E7-TRIF). (C) HEK293 cells (4×10^5) were transfected as described above. Values are means ± standard deviations for three independent experiments. Significances of differences are as follows: c and d, $P < 0.01$; e and f, $P < 0.001$; a and b, $P < 0.0001$.

HI assay. Sera were collected 2 weeks after the booster immunization with pGA-GFP, CMV9-HA, or CMV9-HA-TRIF. Hemagglutinin inhibition (HI) titers were determined by using influenza virus A/PR/8/34, as described previously (4).

Tumor transplantation. Three days before the primary immunization with pGA-GFP, pGA-GFP-E7, or pGA-GFP-E7-TRIF, the mice were administered subcutaneously 2.5×10^4 cells of TC-1, a mouse lung carcinoma expressing E7 antigen. The size of the local tumor mass was monitored for the next 4 weeks.

Statistical analysis. All experiments were repeated at least twice. Statistical significance was evaluated by Student's *t* test, a one-way analysis of variance using the Bonferroni method, or nonparametric survival analysis using the Kaplan-Meier method, with a *P* value of <0.05 considered statistically significant.

RESULTS

Activation of NF-κB and IFN-β promoters by overexpression of TLR adaptor molecules. To identify candidate molecules that might serve as adjuvants for DNA vaccines, a variety of TLR adaptor molecules, including MyD88, TRIF, TIR domain-containing adaptor protein/MyD88 adaptor-like protein (TIRAP/Mal), Toll-interacting protein (TOLLIP), IRAK1, and TRAF6, were tested for the ability to activate the NF-κB and IFN-β promoters of HEK293 cells. NF-κB up-regulates the expression of cytokine, chemokine, and costimulatory molecules central to activation of the innate immune system. Type I IFNs, such as IFN-α and IFN-β, play critical roles in the innate immune response. As shown in Fig. 1A, MyD88 was the most potent inducer of NF-κB activation, while TRIF induced the strongest activation of the IFN-β promoter (*P* < 0.0001). Thus, these two molecules were selected for further evaluation as candidate genetic adjuvants.

Activity of dual-promoter plasmids encoding antigen plus MyD88 or TRIF. To optimize analysis of their adjuvant activity, dual-promoter plasmids were constructed to coexpress LacZ (a surrogate antigen) plus MyD88 or TRIF (Fig. 1B) in a single backbone. Our dual-promoter strategy ensures that the TLR adaptor molecules and an antigen are simultaneously present in the same cells. The ability of these combination plasmids to promote antigen expression was examined in HEK293 cells. Transfection with pGA-LacZ-MyD88 resulted in a 7.8-fold increase in NF-κB-dependent LUC activity compared to control plasmid (pGA-LacZ) (Fig. 1C) (*P* < 0.0001). Transfection with pGA-LacZ-TRIF resulted in an 11.7-fold increase in IFN-β promoter-dependent LUC activity compared with the control plasmid (Fig. 1C) (*P* < 0.001). The levels of LacZ expression were similar in all groups (Fig. 1C). These data demonstrate that antigen expression is comparable among the groups, but innate cell signaling is activated in cells transfected with the combination plasmids of LacZ and a TLR adaptor molecule.

The MyD88-expressing DNA vaccine enhances humoral immunity. Mice were immunized and boosted 4 weeks later by intramuscular electroporation (imEPT) with the pGA-LacZ, pGA-LacZ-MyD88, or pGA-LacZ-TRIF DNA vaccine. Four weeks after primary immunization, all three groups developed similarly low immunoglobulin G (IgG) anti-LacZ Ab responses (Fig. 2A). By comparison, animals immunized and boosted with pGA-LacZ-MyD88 generated sevenfold-higher serum IgG anti-LacZ titers at week 6 than did recipients of the control pGA-LacZ or pGA-LacZ-TRIF plasmid (Fig. 2A) (*P* < 0.01). This improved humoral response was primarily due to the 15-fold-higher IgG2a anti-LacZ response elicited by pGA-
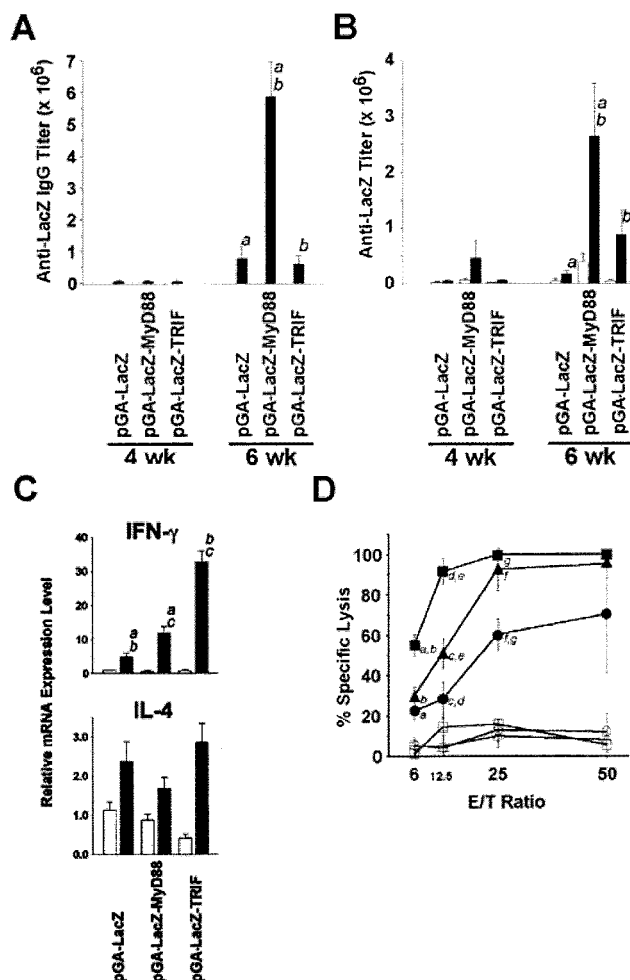

FIG. 2. MyD88 enhances humoral immunity, whereas TRIF enhances cellular immunity. (A and B) Five mice/group were immunized at 0 and 4 weeks with pGA-LacZ, pGA-LacZ-MyD88, or pGA-LacZ-TRIF by imEPT. Blood was drawn at 4 and 6 weeks, and serum anti-LacZ Ab titers were monitored by ELISA. IgG (panel A), IgG1 (panel B, open bars), and IgG2a (panel B, filled bars) titers were examined by ELISA. (C) Splenocytes were prepared from individual mice and stimulated in vitro with 1 μg/ml of class I (H-2^d) peptide (filled bars) or class I (H-2^b) peptide (open bars) for 24 h. mRNA was extracted and reverse transcribed. Aliquots of the reaction were examined for IFN-γ, IL-4, and 18S rRNA levels. The levels of IFN-γ and IL-4 mRNA expression were normalized to the levels of 18S rRNA and described as relative levels of mRNA expression. The graphs present means ± standard deviations. (D) Effector CTL isolates were prepared as described in Materials and Methods. MHC haplotype-matched (H-2K^d) P815 cells were pulsed with 1 μg/ml of class I (H-2^d) peptide (filled symbols) or class I (H-2^b) peptide (empty symbols) for 1 h and used as target cells. The effector and target cells were mixed at effector-to-target ratios of 6 to 50 and cultured for 4 h, and the lactate dehydrogenase activity of the culture supernatants was assayed. The graph shows the mean percent specific lysis ± standard deviation calculated as described in Materials and Methods. ● and ○, pGA-LacZ; ▲ and △, pGA-LacZ-MyD88; ■ and □, pGA-LacZ-TRIF. Significances of differences are as follows: *a* (B), *b* (B), *a* (C), and *c* (C), *P* < 0.05; *a* (A), *b* (A), *b* (C), *c* (D), and *f* (D), *P* < 0.01; *b* (D) and *e* (D), *P* < 0.001; *a* (D), *d* (D), and *g* (D), *P* < 0.0001.

TABLE 1. Characterization of draining LN cells present after vaccine administration

Cell type	No. of cells (mean ± SD) after indicated treatment[a]		
	None	pGA-GFP	pGA-GFP-TRIF
CD11c^{+}	1.01 ± 0.21*†	2.60 ± 0.41*	3.36 ± 0.50†
CD40^{+}	12.68 ± 2.66§	16.58 ± 4.62¶	36.49 ± 4.41§¶

[a] Significances of differences are as follows: *, §, and ¶, $P < 0.01$; †, $P < 0.001$.

LacZ-MyD88 (Fig. 2B) ($P < 0.05$). These results suggest that overexpression of MyD88 improves induction of Th1-dependent antigen-specific humoral immunity to an antigen coexpressed by the same DNA vaccine.

Cellular immune responses modulated by the MyD88 and TRIF genetic adjuvants. The antigen-specific cytokine production by bulk splenocytes from mice 2 weeks post-boost was analyzed. Splenocytes were restimulated in vitro with a LacZ-derived H-2^d-restricted MHC-I peptide, and mRNA levels for IFN-γ and IL-4 were quantified by RT-PCR. Splenocytes from the pGA-LacZ-MyD88- and pGA-LacZ-TRIF-treated groups produced 2.4- and 6.6-fold more IFN-γ mRNA than cells from the control group (Fig. 2C) (P, <0.05 and <0.01, respectively). There was no difference between groups with respect to IL-4 mRNA expression (Fig. 2C). Finally, CTL activity was examined by the lysis of P815 (H-2K^d) cells pulsed with a class I LacZ peptide. CTL isolates from pGA-LacZ-TRIF-vaccinated

animals showed the strongest lytic activity (Fig. 2E) (P, <0.001 at all effector-to-target ratios), although those from pGA-LacZ-MyD88-vaccinated mice also exceeded the response of controls (Fig. 2D) ($P < 0.01$). These results indicate that MyD88 and TRIF can act as genetic adjuvants to enhance the induction of a Th1-dominated cellular immune response to a coexpressed antigen encoded by a DNA vaccine.

TRIF genetic adjuvant increases the number of CD11c$^+$ and CD40$^+$ cells in draining LNs. The immune response induced by DNA vaccines is dependent on transfected antigen-presenting cells (APCs) reaching the draining LNs (1, 5, 20). A single-cell suspension was prepared from inguinal LNs 3 days after boosting, and cell frequencies were determined by surface staining. Compared with resting LNs, the number of CD11c$^+$ dendritic cells (DCs) increased significantly within 72 h of imEPT delivery of plasmid (Table 1) ($P < 0.01$). The pGA-GFP-TRIF plasmids significantly increased the number of CD40$^+$ cells present in draining LNs compared with control plasmid, suggesting that these genetic adjuvants might increase the number of mature B lymphocytes, activated T lymphocytes, and CD40$^+$ DCs at that site (Table 1) ($P < 0.05$).

Effect of TRIF genetic adjuvant on cytokine, chemokine, and cell surface molecule expression in draining LNs. Inguinal LNs were removed 24 and 72 h after booster immunization, and changes in mRNA expression for various cytokines, chemokines, and cell surface molecules were quantified by RT-PCR. The levels of IFN-γ and Th1 cell-promoting cytokines,
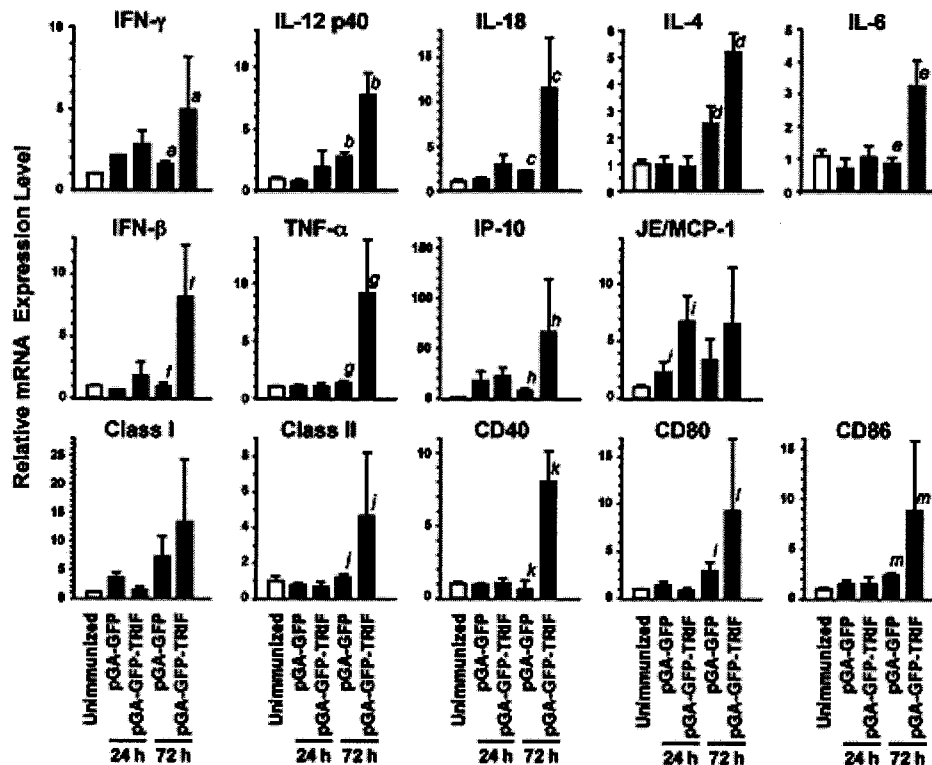

FIG. 3. Activation of cells present in draining LNs after imEPT with the MyD88 or TRIF genetic adjuvant. Twenty-four or 72 h after booster immunization with 50 μg of pGA-GFP or pGA-GFP-TRIF by imEPT, draining LNs (inguinal LNs, 10/group) were removed. Sample mRNA was extracted and reverse transcribed, and then the cDNA aliquots were assessed for expression of IFN-γ, IL-12 p40, IL-18, IL-4, IL-6, IFN-β, TNF-α, IP-10, JE/MCP-1, MHC-I, MHC-II, CD40, CD80, and CD86 by RT-PCR. The graph shows means ± standard deviations for samples prepared from 10 individual LNs. Significances of differences are as follows: a, d, i, j, l, and m, $P < 0.05$; b, c, e, f, g, h, and k, $P < 0.01$.
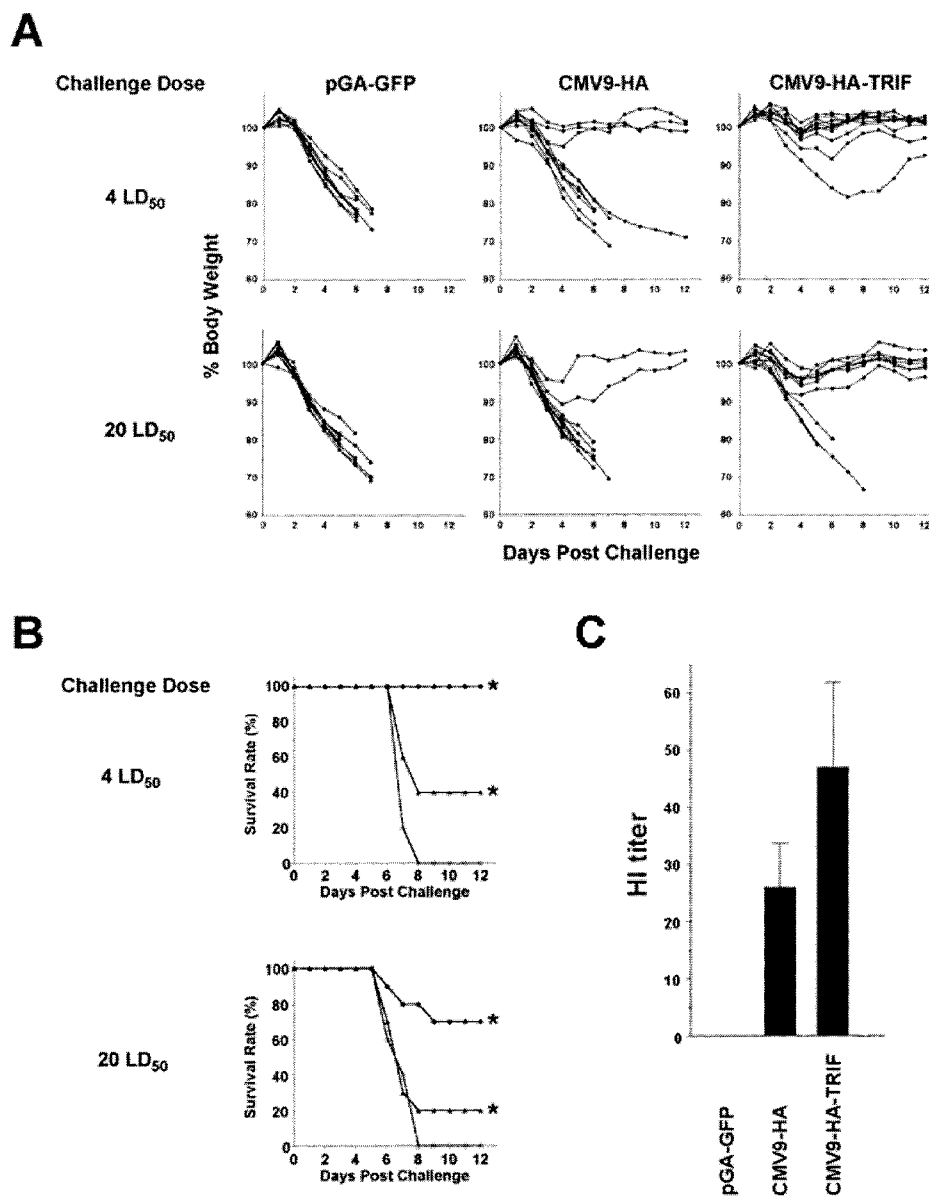
FIG. 4. TRIF increases the protective activity of an influenza HA-encoding DNA vaccine. Ten mice/group were immunized at 0 and 4 weeks with 2 μg of pGA-GFP, CMV9-HA, or CMV9-HA-TRIF by imEPT. Two weeks later, they were challenged with 4 or 20 LD_{50} of influenza virus A/PR/8/34. (A) Percent change in body weight compared to day zero. (B) Survival rates monitored for the following 12 days. ○, pGA-GFP; ▲, CMV9-HA; ●, CMV9-HA-TRIF. ∗, $P < 0.01$. (C) HI titers measured with sera obtained 2 weeks after booster immunizations.

including IL-12 p40 and IL-18, were significantly up-regulated 72 h after immunization with pGA-GFP-TRIF compared to control groups (Fig. 3) ($P < 0.05$). Th2 cytokine mRNAs, including IL-4 and IL-6, were also up-regulated at 72 h ($P < 0.05$). Factors regulating APC function (i.e., IFN-β, TNF-α, IP-10, and JE/MCP-1) and elements associated with APC function (i.e., MHC-II, CD40, CD80, and CD86) were consistently increased 72 h after immunization with pGA-GFP-TRIF compared to control plasmid (Fig. 3) ($P < 0.05$).

Incorporation of TRIF genetic adjuvant into DNA vaccines targeting influenza and tumors. To explore whether TRIF could contribute to improving the protective effect of a DNA vaccine, vectors targeting the influenza HA antigen and the

tumor-associated antigen E7 were constructed (Fig. 1B). Mice primed and boosted with each HA-encoding vaccine were challenged intranasally 2 weeks later with 4 to 20 LD_{50} of live influenza virus A/PR/8/34. Two outcomes of disease were monitored: weight loss and mortality. Animals immunized with the irrelevant pGA-GFP plasmid uniformly demonstrated severe weight loss and subsequently died (Fig. 4A and B). Vaccination with CVM9-HA protected approximately half of the mice from low-dose challenge but <20% of animals from higher-dose challenge. In contrast, the CMV9-HA-TRIF vaccine protected all mice from low-dose challenge and 70% of mice from high-dose influenza virus challenge (P < 0.05). The HI titer was determined as shown in Fig. 4C. CMV9-HA-TRIF and
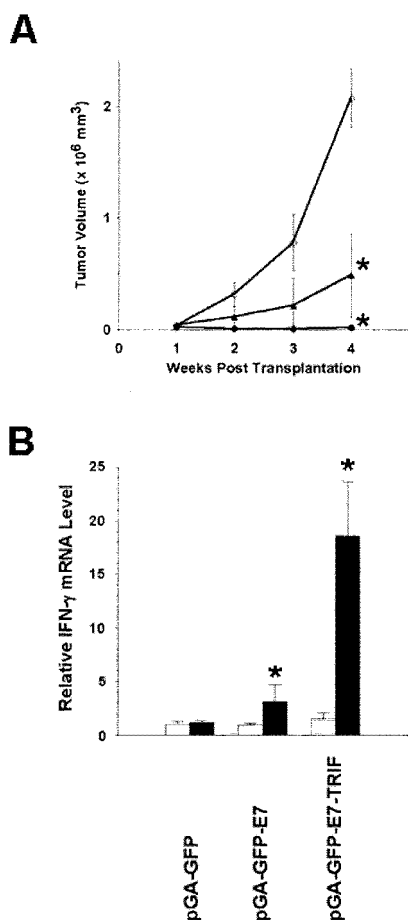
FIG. 5. TRIF increases the protective activity of a tumor-associated antigen E7-encoding DNA vaccine. (A) Three days before vaccination, mice were challenged with 2.5×10^4 TC-1 cells. Ten mice/group were immunized at 0 and 2 weeks with 20 μg of pGA-GFP, pGA-GFP-E7, or pGA-GFP-E7-TRIF by imEPT. Tumor outgrowth was monitored for the following 4 weeks. ○, pGA-GFP; ▲, pGA-GFP-E7; ●, pGA-GFP-E7-TRIF. *, $P < 0.05$. (B) Splenocytes were prepared 2 weeks after the booster immunization and stimulated in vitro with 1 μg/ml of E7 peptide (filled bars) or NP peptide (open bars) for 24 h. The relative IFN-γ mRNA expression levels were measured as described in the legend to Fig. 2. *, $P < 0.01$. The graphs present means ± standard deviations.

CMV9-HA raised comparable levels of HI activity, suggesting that the TRIF genetic adjuvant raised a stronger innate and/or cellular immune response, which may confer superior protection in combination with the humoral immune response (Fig. 4C). Mice that had been established with subcutaneous tumors were primed and boosted with each E7-expressing vaccine. Tumor masses grew steadily in mice vaccinated with the irrelevant pGA-GFP plasmid (Fig. 5A). pGA-GFP-E7 treatment partially protected against tumor outgrowth, while pGA-GFP-E7-TRIF progressively protected (Fig. 5A). This observation was in accordance with the levels of E7-specific IFN-γ production from splenocytes of immunized mice (Fig. 5B). These results clearly indicate that the TRIF genetic adjuvant can improve the protective effect of DNA vaccines in infectious

and neoplastic disease models, both major targets of current vaccine development.

DISCUSSION

There is an urgent need to develop effective vaccines against emerging infectious diseases and biothreat pathogens (12, 13). DNA vaccines represent a promising strategy for accomplishing this goal, but their success has been limited due to poor immunogenicity in large animals. We postulated that TLR adaptor molecules might be used as genetic adjuvants to promote immunity against DNA vaccine-encoded antigens by their nature as activators of innate immune responses. To minimize potential side effects, these agents were incorporated into the antigen-encoding plasmid, thereby insuring their targeting to cells contributing to the induction of protective immunity. Initial results demonstrated that both MyD88 and TRIF were functionally active, enhancing antigen-specific humoral and/or cell-mediated immune responses in vivo. Of greater importance, the addition of TRIF significantly improved the protective activity of an HA-encoding DNA vaccine in mice challenged with influenza virus and the therapeutic activity of an E7-encoding DNA vaccine in mice transplanted with lung carcinoma cells.

Incorporating cassettes expressing the MyD88 or TRIF gene into DNA vaccine vectors increased the size of the DNA by 2.1 and 3.7 kb, respectively. Since the size of the DNA vaccine generally affects in vivo transfection efficiency (6, 15), plasmids containing the genetic adjuvant cassettes were less-effective inducers of target molecule expression (data not shown). Since IFNs and proinflammatory cytokines have the ability to suppress virus promoters, such factors induced by genetic adjuvants might inhibit the levels of antigen expression. However, DNA vaccine immunogenicity was improved by increasing the expression of MyD88 or TRIF in transfected cells. MyD88 plays an essential role in DC maturation, thereby improving CD8+ T-lymphocyte priming via cross-presentation (18). MyD88-deficient mice have a profound defect in the activation of antigen-specific Th1 immune responses (23). TLR3-deficient mice exhibit impaired CTL cross-priming of antigens associated with virus-infected cells, indicating that TLR3-mediated TRIF-dependent signaling provides an important link between innate and acquired immunity following virus infection (24). Type I IFNs play a key role in maintaining innate and acquired immunity. Mice lacking type I IFN receptors or signaling molecules exhibit defective immune responses to eliminate cancer cells and cells infected by viruses (32). Recent studies have demonstrated that TLR-mediated cellular signaling is also mediated by IRF-3 or IRF-7, which serves as a crucial transcription factor regulating the production of IFN-β or IFN-α, respectively (9). In this regard, we demonstrated previously that IRF-3 and IRF-7 genetic adjuvants are both effective at amplifying DNA vaccine-raised immune responses (22).

A numbers of cytokines, hematopoietic factors, and costimulatory molecules have been tested for the ability to act as genetic adjuvants (25, 33, 34). However, the immunological processes associated with stimulation through these molecules are downstream of the activation of the innate immune system mediated by MyD88 and TRIF following TLR ligation. We therefore hypothesized that increasing expression of these molecules would im-

prove the signaling cascade required for activation of both innate and subsequent adaptive immune responses.

In conclusion, the present study demonstrates that TLR adaptor molecules, particularly TRIF, can improve the immunogenicity and protective and therapeutic effects of DNA vaccines. These findings may open a new avenue for designing vaccines that elicit both innate and adaptive immunity, mimicking the effects of live pathogen exposure. Following up the present study with further vaccination studies that involve TRIF genetic adjuvant targeting of a variety of microbial and tumor antigens is necessary to realize the value of this novel approach.

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